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Na artelinate and Na artesunate were active against qinghaosu-resistant parasites. Halofantrine was active against mefloquine and chloroquine-resistant parasites. Against drug-sensitive parasites arteether was more active than a tetraoxane (BM 07749) followed by Na artelinate and artemisinin, however, arteether was toxic at higher doses. Of two bisquinolines (BM 03821) was more active and less toxic than BL 57511. Synergistic activity was observed between WR 238417, the putative biguanide precursor of a triazine (WR 99210), and both sulfadiazine and pyrimethamine against pyrimethamine-resistant parasites. Louderback Sterilizing Medium killed parasites in vitro at various inoculum levels up to 5000X the regular MM test inoculum. Qinghaosu cleared the parasites from the peripheral blood by 35 hrs when given once SC on day 3, however, by day 10 they recrudesced. The WR 238605-resistant line has remained stable under drug pressure for this year. Co-enzyme Q_{10} did not act as an antioxidant like vitamin E during a malarial infection. The oils, MCT and Miglyol, were found to be suitable placebos for fish oil. A normal chow diet (with adequate vitamin E levels) supplemented with 20% omega-3 fish oil suppressed the growth of malarial parasites. Lipid peroxidation byproduct in the urine of mice maintained on omega-3 diet deficient in vitamin E did increase which showed oxidative stress maybe an important mechanism involved in killing malarial parasites.

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__✓__In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use for Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animals Resources. National Research Council (NIH Publications No. 86-23, Revised 1985).

_____For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45 CFR 46.

_____In conduction research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

PI Signature Carla Z Ozer Date 11/27/91

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ABSTRACT

In the primary blood schizonticidal test 1,105 compounds were evaluated and 39 exhibited antimalarial activity. Dihydroartemisinin cured all mice when administered in a total dose of 100 mg/kg given PO for 4 days (D+0, 1, 2 and 3) at 12 hr intervals. A 50 mg/kg total dose administered in a similar regimen cured only 1 of 10 mice when administered PO at 8 hr intervals for 5 days (D+0, 1, 2, 3 and 4). A total dose of 60 mg/kg of Na artelinate and Na artesunate were inactive while dihydroartemisinin at 240 mg/kg was curative. Two tetraoxanes (BM 07749 and BM 07721) given PO on days 3, 4 and 5 cured all mice at 640 and 1280 mg/kg/day. Transdermal applications of artemisinin and 3 analogs in a prophylactic regimen (D+0, 1 and 2) or a curative regimen (D+3, 4 and 5) showed dihydroartemisinin to be the most active followed by artelinic acid, methyl artelinate and artemisinin. All of the transdermal drugs had penetrated by 30 minutes as judged by cleaning the treated area with alcohol. In the Thompson test 35 experiments were performed to assess suppressive and curative activity of compounds against drug-sensitive lines resistant or lines resistant to either qinghaosu, WR 238605, mefloquine, chloroquine, pyrimethamine or quinine. Na artelinate and Na artesunate were active against qinghaosu-resistant parasites. Halofantrine was active against mefloquine and chloroquine-resistant parasites. Against drug-sensitive parasites arteether was more active than a tetraoxane (BM 07749) followed by Na artelinate and artemisinin, however, arteether was toxic at higher doses. Of two bisquinolines (BM 03821) was more active and less toxic than BL 57511. Synergistic activity was observed between WR 238417, the putative biguanide precursor of a triazine (WR 99210), and both sulfadiazine and pyrimethamine against pyrimethamine-resistant parasites. Louderback Sterilizing Medium killed parasites *in vitro* at various inoculum levels up to 5000X the regular MM test inoculum. Qinghaosu cleared the parasites from the peripheral blood by 35 hrs when given once SC on day 3, however, by day 10 they recrudesced. The WR 238605-resistant line has remained stable under drug pressure for this year. Co-enzyme Q_{10} did not act as an antioxidant like vitamin E during a malarial infection. Two oils, MCT and Miglyol, were found to be suitable placebos for fish oil. A normal chow diet (with adequate vitamin E levels) supplemented with 20% omega-3 fish oil suppressed the growth of malarial parasites. Lipid peroxidation byproducts in the urine of mice maintained on omega-3 diet deficient in vitamin E did increase which showed oxidative stress maybe an important mechanism involved in killing malarial parasites.

INTRODUCTION

Today malaria is actively transmitted in 102 countries in the World where it infects over 400 million people of which 100 million have clinical cases often resulting in severe morbidity and culminating in over 2 million annual deaths. This presents a major problem to non-immune people, especially U.S. military personnel and civilians, who venture into these endemic areas. *Plasmodium falciparum* is the most pathogenic species of the four occurring in humans and the number of cases caused by this species is rising each year. The major problem with *P. falciparum* is its resistance to chemotherapeutic agents. Often multidrug-resistant strains are encountered rendering treatment ineffective. Recent reports of chloroquine-resistant *Plasmodium vivax* have been substantiated and pose a future major obstacle if disseminated in other endemic areas. Other major problems encountered in controlling malaria include mosquito resistance to insecticides, and a lack of malaria vaccine. These two obstacles may never be overcome leaving the control and prevention of malaria to chemotherapeutic agents. The only two new drugs emerging since the 1950's to combat chloroquine-resistant and often multiple drug-resistant *P. falciparum* are mefloquine and halofantrine. Resistance in some *P. falciparum* parasites has been found to both of these drugs and unfortunately there are reports of toxicity problems emerging with mefloquine.

The Institute of Medicine, a part of the National Academic of Science, just published a report of their committee for the study on malaria prevention and control: Review and Alternative Strategies. The report was entitled "Malaria Obstacles and Opportunities" and contained in its conclusions and recommendations section the following statement; "The committee recommends increased emphasis on screening compounds to identify new classes of potential antimalarial drugs, identifying and characterizing vulnerable targets within the parasite, understanding the mechanisms of drug resistance, and identifying and developing agents that can restore the therapeutic efficacy of currently available drugs." It is unfortunate that drug companies are not actively seeking to identify new antimalarial agents. Because of this need to identify new classes of potential antimalarial drugs we are currently testing 1,000 compounds per year against drug-sensitive malaria in a standardized primary antimalarial test system (MM Test).

Selected active compounds emerging from this MM test are further evaluated in a secondary test system (AG Test) for suppressive and curative activity. These specialized studies include 1) activity against malarial parasites resistant to one of the following drugs, chloroquine, mefloquine, quinine, qinghaosu, pyrimethamine, dapsone, cycloguanil or WR 238605; 2) multiple dosing regimens; 3) detecting synergistic activity between compounds; 4) induction of resistance to specific compounds in a systematic method; 5) determining the best route, vehicle, and time to administer a compound; 6) studying the influence of the hosts antioxidant status with the hope of potentiating the antimalarial activity of selected drugs.

PROCEDURE FOR ASSESSING THE BLOOD SCHIZONTICIDAL ANTIMALARIAL ACTIVITY OF CANDIDATE COMPOUNDS IN *PLASMODIUM BERGHEI* INFECTED MICE

This mouse malaria (MM) test system was designed to identify new compounds active against asexual blood stages of malaria. Using mice from our breeding colony and a standard inoculum of *P. berghei* it has been possible to produce a consistent disease fatal to 100% of the untreated animals within 6 to 7 days. Active compounds extend the survival time or cure infected mice.

An established disease is less responsive to treatment than a disease in the early stages of development, therefore treatment was deliberately withheld until a moderately high degree of parasitemia was evident. Test compounds were administered subcutaneously (SC) in a single dose on the third day postinfection, at which time a 10-15% parasitemia had developed. A similar procedure was followed for the oral (PO) administration of selected active compounds.

A compound was classified as "active" if it suppressed the disease and produced and unquestionably significant increase, 100% or more, in the life span of the treated animals over that of the untreated infected controls. A compound was considered to be "curative" if the treated animals remained alive for 60 days after infection with *P. berghei.* Compounds not meeting one of the above requirements were considered "inactive".

The severity of the challenge set up in the MM test system enhances the reliability of our evaluation and the antimalarial potential of the compounds selected for intensive preclinical studies.

METHODS

ANIMAL HOSTS

The total supply of animals needed to test candidate compounds was obtained from our breeding colony of CD-1 Swiss mice (*Mus musculus*). Test animals weighed 18-20 grams. Weight variations in any given experimental or control group were carefully limited to within 2 to 3 grams. In any given test all animals were approximately the same age.

Animals on test were housed in metal-topped plastic cages, fed a standard laboratory diet and given water *ad libitum*. Once the infected mice had been administered the drug, they were placed in a room maintained at 28.8°C(±2°C), with a relative humidity of approximately 66%.

TEST PROCEDURE

Test animals received an intraperitoneal (IP) injection of approximately $6X10^5$ parasitized erythrocytes drawn from donor mice infected 4 days earlier with *P. berghei*. The donor strain was maintained by passage every 4 days in separate groups of mice inoculated with 0.2 cc of a 1:435 dilution of heparinized heart blood.

To check factors such as changes in the infectivity of our *P. berghei* strain or in the susceptibility of the host, 1 group of mice, which served as the negative control, was infected but not treated. To determine the effect that a drug exerted upon a malarial infection, 2 parameters were measured; the first was an increase in survival time, the second concerned curative action. For comparative purposes, 1 standard compound, pyrimethamine, was administered at 1 level (120 mg/kg) to a group of 15 mice. Pyrimethamine served as a positive control, producing a definite increase in survival time and curative effects. Another function of the positive control involved monitoring 3 procedures; the drug weighing, the preparation of drug solutions and suspensions, and the administration of drugs.

DRUG ADMINISTRATION

Test compounds were dissolved or suspended in peanut oil before they were administered SC. Compounds to be administered PO were mixed in an aqueous solution of 0.5% hydroxyethylcellulose-0.1% Tween-80 (HEC).

Treatment consisted of a single dose given SC or PO 3 days postinfection. At the time of treatment a 10-15% parasitemia had developed. Although the disease was well established, it had not yet caused sufficient debility to affect an evaluation of the test compound's toxicity.

Deaths that occurred before the sixth day, when untreated infected controls began to die, were regarded as the result of a compound's toxic effect and not as the result of action by the infecting parasite.

Each compound was initially administered in 3 graded doses, diluted 4-fold, to groups of 5 mice per dose level. The top dose was 640, 320, or 160 mg/kg of body weight depending upon the amount of compound available for testing. Active compounds were subsequently tested at 6 or 9 dose levels, diluted 2-fold from the highest dose. Successive 6-level tests were performed at respectively lower doses until the lower limit of activity was reached, thus establishing a complete dose-response picture for that compound in a rodent system.

A drug that was toxic for the host at each of the 3 levels initially tested was retested at 6 dose levels diluted 2-fold from the lowest toxic dose.

RESULTS

During this year 1,105 compounds were tested for activity against asexual blood stages of *P. berghei*. There were 39 of these compounds which exhibited antimalarial activity.

A total of 301,418 3-dose level tests were performed from December 1, 1961 through September 30, 1991 (**Table I**). The number of active compounds during part of this time period is summarized in **Table II**.

The specific test data of all compounds test, (in all malaria drug tests) many of which are commercially or proprietorial discreet, is transmitted to the WRAIR Drug Development Program Chemical/Biological database.

TABLE I

PLASMODIUM BERGHEI MALARIA IN MICE

COMPOUNDS TESTED

DECEMBER 1, 1961 - SEPTEMBER 30, 1991

TIME PERIOD	NUMBER OF COMPOUNDS TESTED
October 1, 1990 - September 30, 1991	1,105
February 1, 1990 - September 30, 1990	222
February 1, 1989 - January 31, 1990	1,501
February 1, 1988 - January 31, 1989	1,502
February 1, 1987 - January 31, 1988	1,500
February 1, 1986 - January 31, 1987	1,507
February 1, 1985 - January 31, 1986	1,500
October 1, 1983 - January 31, 1985	3,390
October 1, 1982 - September 30, 1983	3,026
October 1, 1981 - September 30, 1982	3,020
October 1, 1980 - September 30, 1981	2,998
October 1, 1979 - September 30, 1980	4,826
October 1, 1978 - September 30, 1979	6,175
October 1, 1977 - September 30, 1978	5,375
June, 1974 - September, 1977	27,634
December 1, 1961 - May, 1967	76,853
TOTAL	301,418

TABLE II

PLASMODIUM BERGHEI MALARIA IN MICE

SUMMARY OF ACTIVE COMPOUNDS

JUNE 1, 1970 - SEPTEMBER 30, 1991

TIME PERIOD	NUMBER OF COMPOUNDS TESTED	NUMBER OF ACTIVE COMPOUNDS
October 1, 1990 - September 30, 1991	1,105	39
February 1, 1990 - September 30, 1990	222	47
February 1, 1989 - January 31, 1990	1,501	39
February 1, 1988 - January 31, 1989	1,502	167
February 1, 1987 - January 31, 1988	1,500	327
February 1, 1986 - January 31, 1987	1,507	158
February 1, 1985 - January 31, 1986	1,500	74
October 1, 1983 - January 31, 1985	3,390	205
October 1, 1982 - September 31, 1983	3,026	335
October 1, 1981 - September 31, 1982	3,020	574
October 1, 1980 - September 31, 1981	2,998	359
October 1, 1979 - September 31, 1980	4,826	581
October 1, 1978 - September 31, 1979	6,175	969
October 1, 1977 - September 31, 1978	5,375	1,261
June 1, 1976 - September 31, 1977	7,144	1,124
June 1, 1970 - May 31, 1976	78,713	3,530
TOTAL	123,504	9,789

SECONDARY ANTIMALARIAL TEST SYSTEM

INTRODUCTION

DRUG RESISTANCE

Many *P. falciparum* parasites in various geographic areas of the world do not respond to certain standard antimalarial agents while some of these parasites do not respond to any antimalarial agent (multiple drug resistance). The different categories of drug resistance found in *P. falciparum* are summarized below;

1) Resistance to 4-aminoquinolines
   chloroquine

2) Resistance to arylaminoalcohols
   mefloquine (a quinolinemethanol)
   halofantrine (a phenanthrenemethanol)

3) Resistance to cinchona alkaloids
   quinine

4) Resistance to antifol drugs
   pyrimethamine
   proguanil
   Fansidar^{R}
   Fansimef^{R}

5) Resistance to acridines
   atebrine

6) Multiple drug resistance (resistance to two or more of the above compounds).

DRUG ACTIVITY

TOXICITY

Toxic reactions in humans can occur with many antimalarials. The following compounds have been shown to cause severe toxic reactions in some patients.

Primaquine
Amodiaquine
Fansidar^{R}
Fansimef^{R}
Mefloquine
Quinine

RECRUDESCENCE

The emergence of recrudescent parasites is an important factor in approximately 20% of patients treated with artemisinin and several of its analogs which have been used in China. Therefore, other drugs need to be administered to obtain cures. The main advantages of sesquiterpene lactones is their activity against chloroquine-resistant *P. falciparum*, their fast action and low toxicity, and lack of artemisinin-resistant parasites in nature.

SLOW ACTING COMPOUNDS

Several antibiotics (tetracycline, doxycycline, minocycline, and clindamycin) have been used against malarial infections in humans but when used alone they act very slow in clearing the parasites so they are not better than other antimalarials against drug-sensitive parasites. Their main advantage is when combined with other drugs against multidrug-resistant parasites.

SYNERGISTIC COMBINATION

Two synergistic combinations used today Fansidar^{R} (pyrimethamine, sulfadoxine) and Fansimef^{R} (pyrimethamine, sulfadoxine, and mefloquine) are no longer recommended because of toxicity due to sulfadoxine and also the development of resistance to them. Synergistic drug combinations in theory have two major advantages, the first being less drug of each component is needed to obtain curative activity and combination therapy is reported to slow down the emergence of resistant parasites. Currently CDC recommends a drug regimen composed of proguanil and doxycycline for chloroquine-resistant *P. falciparum* infections. This is not a satisfactory therapy for such parasites because there is resistance to proguanil and doxycycline is a slow acting antibiotic.

The currently available antimalarial drugs are not satisfactory to treat malaria today because of multiple drug resistance, toxicity, recrudescence, and slow antimalarial action. Therefore, new novel chemical classes exhibiting antimalarial activity are needed

and other novel ways to combat the evasive malarial parasites. Such new methods include reversing chloroquine resistance, transdermal application of antimalarial agents, finding new synergistic drug combination, alteration of the hosts antioxidant system and fatty acid profile of parasite membranes rendering them more susceptible to prooxidant drugs.

The antioxidant status of the hosts blood system is an important component often influencing the degree of parasitemia and the eventual pathology caused by malarial organisms. These natural antioxidants may also antagonize the antimalarial activity of drugs which act via free radical formation as their primary mechanism in killing malarial parasites. Such drugs acting through the generation of free radicals include peroxides, trioxanes, tetraoxanes, primaquine, qinghaosu-type compounds, and possibly WR 238605.

By manipulating the hosts major antioxidative component in the blood (vitamin E) in concert with metabolically shifting the fatty acid profile of red blood cell and parasite membranes to an increased level of polyunsaturated (PUFA) omega-3 (n-3) fatty acids, one could render the infected red blood cells and the parasite more susceptible to killing by free radical acting drugs.

METHODS

PARASITES

Drug-sensitive lines

Plasmodium berghei KBG-173 (P-line)
Plasmodium yoelii 17 X Lethal (Py-line)
Plasmodium yoelii 17 X non-lethal (Py NL-line)
Plasmodium vinckei (PV-line)

Drug-resistant lines

P. berghei KBG-173

mefloquine resistant	A-line
chloroquine resistant	C-line
pyrimethamine resistant	M-line
dapsone resistant	S-line
cycloguanil resistant	T-line

P. yoelii 17 X

qinghaosu resistant	QHS-R
WR 238605 resistant	8AQ-R

P. vinckei

chloroquine resistant	PV-CR
quinine resistant	Q-line

ANIMAL HOST

The testing was done in both female and male CD-1 Swiss mice (*Mus musculus*) obtained from our own breeding colony. Four week-old mice were used for most experiments except 3 week-old weanling male mice were used to start the antioxidant studies.

TEST DESIGN

MULTIPLE DOSE MODIFIED MM TESTS

Dihydroartemisinin was administered PO for 4 days (D+0,1,2,3 postinfection) b.i.d. at 12 hr intervals to mice infected with a regular MM parasite inoculum of $6X10^5$ erythrocytes parasitized with *P. berghei*. Blood films were taken to assess suppressive activity and survival times were monitored for 60 days to assess curative activity (Exp.46). Artesunate, Na artelinate and dihydroartemisinin were administered PO for 5 days (D+0,1,2,3,4) b.i.d. at 8 hr intervals to mice infected with a regular MM inoculum (Exp.47). Chloroquine was administered PO at 8 hr intervals on days 0 and 1 to mice infected with a regular MM inoculum of chloroquine-resistant parasites (Exp.59). Three tetraoxanes (BM 07749 and 2 other related compounds) were administered SC for 3 days (D+3,4,5) once a day to mice infected with a regular MM inoculum (Exp.61). BM 07749 was administered in Exp.66 at higher doses in a similar plan than was done in experiment 61. In experiment 69 BM 07749 was tested again along with BM 07721 and BM 07730 just like the tetraoxanes in experiment 61.

Two experiments were designed to test compound MA 680 in mice infected with a regular MM inoculum. In experiment 63 the compound was administered PO b.i.d. on days 3,4, and 5 postinfection. Higher doses of the compound (MA 680) were administered to mice in experiment 65 in a similar procedure than that used in experiment 63.

TRANSDERMAL ADMINISTRATIONS

Several analogs of qinghaosu were prepared in a gel for application to the skin of mice. These formulations were administered at different times and on different days to shaven mice infected with a regular MM parasite inoculum. Artelinic acid was given for either 3 or 6 days at 8 hr intervals in experiment 48. In experiment 49 artelinic acid was given once a day for either 2, 4 or 6 days. Artelinic acid was administered at 8 hr intervals for either 1, 2 or 3 days postinfection in experiment 50. In experiments 53, 54, 55 and 58 artelinic acid was administered in a gel with or without azone. These formulations were given at different drug levels and intervals postinfection. In experiment 56 artelinic acid was given with azone to mice on days 3,4, and 5 after they had been housed in a room maintained at 75°C for days 0, 1 and 2 then switched to a room at 84°C for the duration of the test. In all other experiments mice were kept in the 84°C room for the entire experiment. Mice infected with chloroquine-resistant parasites were treated with artelinic acid in experiment 60. Artelinic acid was applied to both sides of mice once a day on days 3 and 4 postinfection in experiment 70. Methyl artelinate was compared with methyl artelinic acid on days 0, 1, and 2 or days 3, 4, and 5 in experiment 71. Artemisinin and dihydroartemisinin were administered on days 0, 1, 2 or 3,4,5 to mice in experiment 72. In order to establish the length of time artelinic acid remained on the skin of mice experiment 74 was designed to clean the skin area receiving drug at intervals of 1/2 hr, 1 hr, or 2 hrs post-treatment. Mice were dosed on days 3, 4, and 5 at 8 hr intervals and cleaned at the respective times post-treatment.

In experiment 75 Balb/c mice were treated with artemisinin, dihydroartemisinin, artelinic acid and methyl artelinate on days 3, 4, and 5 postinfection at 8 hr intervals. Low doses of artemisinin and dihydroartemisinin were administered on days 0, 1, and 2 or 3, 4, and 5 postinfection at 8 hr intervals in experiment 76. Similar low doses of artelinic acid and methyl artelinate were given in a similar regime in experiment 78.

THOMPSON SUPPRESSIVE AND CURATIVE TEST

In this Thompson test, mice were divided into groups of 7 and inoculated with usually a standard inoculum of $5X10^4$ parasitized erythrocytes. This inoculum load can vary with drug-resistant lines of parasites. Drugs were administered twice a day, in a volume of 10 ml/kg on the third, fourth and fifth days after inoculation of parasites. Drugs were mixed in HEC for PO administration and in peanut oil for the SC route. One group of infected mice received the vehicle alone and served as a negative control group.

Blood films were made on the sixth day after inoculation of parasites and weekly thereafter for 60 days. Microscopic examination of Giemsa-stained blood smears was made to determine the percentage of cells parasitized. The percent suppression of parasitemia, and significance values for the suppression of parasitemias were then determined. Significance values for the percent suppression of parasitemia for 6-day parasitemias only were determined by comparing the parasitemia of each treated mouse with the mean parasitemia of the negative control animals. Drug tolerance was reflected by the percent weight change and the proportion of mice that survived treatment.

There were 35 test performed in this time period (673-709).

SYNERGISTIC STUDIES

Two studies were done to evaluate if 2 compounds would act synergistically in suppressing and curing malaria infections using the following procedure. The drugs were mixed separately then administered either alone or as a mixture PO twice a day on days 3, 4, and 5 after the mice had been infected. The effects were read from blood smears made 1 day after completion of treatment. The SD_{90} values of the drugs alone and of the mixture were estimated by plotting parasitemia suppressions on probit log scale graphs. The analyses for synergism were based upon partitioning of the SD_{90} value of each combination in terms of its components. These components were then compared with the respective SD_{90} if no interaction occurred the value would be expected to be 0.5. If all values were lower than 0.5 the data would indicate synergism. Conversely, if all values were greater than 0.5 the data would indicate antagonism. Blood films were taken at weekly intervals until day 60 postinfection. Survival times were also monitored for this 60 day period. In experiment 680 WR 250417, the putative biguanide precursor of a triazine (WR 99210), was tested together with either sulfadiazine or pyrimethamine against pyrimethamine-resistant parasites. WR 99210 had poor oral bioavailability and gastrointestinal intolerance when tested in man. Possibly oral administration of the biguanide precursor could elevate these problems allowing the subsequent metabolism to the active triazine resulting in therapeutic levels of this active triazine. In a second synergistic study (experiment 690) N-acetylcysteine was co-administered with quinine in mice inoculated with either quinine-resistant parasites or drug-sensitive parasites. The purpose of this experiment was to see whether N-acetylcysteine would increase the toxicity of quinine because N-acetylcysteine is being tested in humans with cerebral malaria that are being treated quinine. The rationale for using N-acetylcysteine lies in its ability to scavenge cerebral free oxygen radicals which may cause pathology during a malarial infection. It has been shown that tumor necrosis factor levels increase during a malarial infection and stimulate the production of increased levels of dangerous free oxygen radicals that may cause some of the cerebral pathology.

LOUDERBACKS STERILIZING MEDIUM

A solution of formaldehyde and Brij detergent known as Louderback Sterilizing Medium (LSM) which was developed for treatment of virus contaminated blood to be used for blood transfusions, was evaluated for is ability to kill malarial parasites *in vitro*. To verify if all the parasites were rendered non infective a series of experiments were performed to test if the treated infected blood produced a malarial infection when incubated into normal mice. In the first experiment (62) a standard inoculum of MM parasites ($6X10^5$ parasitized erythrocytes) was incubated in LSM for 30 minutes before injecting it into normal mice. In experiment 64 three different parasite inoculums (a regular MM of 1X, then 5X and 10X) were tested. The 10X inoculum level was retested in experiment 68. Higher inoculum levels of 100X, 1000X, and 5000X were tested in experiment 73. Qinghaosu-resistant parasites were treated with LSM in experiment 77. Experiment 79 was repeat of experiment 73.

RATE OF PARASITE CLEARANCE STUDIES

Several experiments were designed to evaluate how fast a drug administered IV, to mice with an established infection, would clear the parasites from the peripheral blood system. In experiment 51 qinghaosu was given SC at 640, 160, and 40 mg/kg and chloroquine was administered at 160 and 80 mg/kg. Artelinic acid was administered at 320, 160, and 80 mg/kg in experiment 65. Chloroquine was tested at 80 and 40 mg/kg in experiment 67.

MAINTENANCE OF ER 238605-RESISTANT LINE THROUGH MOSQUITOES

The new line resistant to WR 238605 was kept under continuous drug pressure and after every second blood passage it was passed through mosquitoes. The regular passage through mosquitoes is needed to maintain its gametocyte population which can be lost with only continuous blood passage. The other reason to pass through mosquitoes was to allow for genetic changes to occur just as happens to malarial parasites in nature. We monitored any changes in susceptibility to standard drugs periodically. The line is given oral doses of 32 mg/kg/day for 3 days on days 3, 4, and 5 after inoculation of asexual blood stages to maintained drug resistance.

The drug-sensitive parent line of WR 238605-resistant line is passess weekly through mosquitoes for the same reason as stipulated above.

ANTIOXIDANT STUDIES INVOLVING CHANGING FATTY ACID PROFILES IN MEMBRANES

A series of experiments were performed to study the influence of altering the fatty acid profiles of host red blood cells and parasites membranes in infected mice fed vitamin E-deficient diets. Various polyunsaturated fatty acids high in n-3 fatty acids were used as the dietary fat source in these diets. Suppressive and curative antimalarial activity was determined by monitoring parasitemia levels and mortality data.

In experiment 73 an ethyl ester concentrate of menhaden oil was fed to mice for 4 weeks prior to infection and continued for only 1 week postinfection. This was done in order to evaluate if suppressive and curative antimalarial activity could be achieved when the mice were switched back to regular mouse chow. Experiment 74 was designed to see if inbred Balb/c mice would respond to this dietary control of malaria just as the outbred CD-1 mice did. The influence of paraaminobenzoic acid (PABA) was also studied. In experiment 75 we examined whether feeding mice a chow diet for 1 week prior to starting them on vitamin E depleted diets would alter their plasma vitamin E levels at 2 or 4 weeks after feeding the vitamin E depleted diets. These mice were not infected with malaria.

In experiment 76 the antioxidant Coenzyme Q_{10} (ubiquinone 50) was tested to see if it would replace vitamin E as an antioxidant and influence the growth of malaria in mice maintained on a vitamin E-deficient diet containing n-3 fatty acids for 1 month. Mice were dosed with either 100, 36, or 3 mg/kg/day for 7 consecutive days commencing 1 day before infection with malaria. Experiment 77 was designed to explain the mechanism involved in the curative activity obtained in mice fed n-3 fatty acid without vitamin E. Other workers have shown antibodies are important in mediating immunity to malaria and that the IgG1a isotype plays a dominant role in modulating the parasitemia values. To study the role of antibody isotypes mice were placed on an n-3 fatty acid diet for 4 weeks then infected with malaria. Blood samples where taken at weekly intervals for 35 days. The mice were rechallenged 207 days later and blood samples were taken from these mice weekly for 35 days. Antibody isotypes were quantitated in the plasma samples by Dr. Diane Taylor (Georgetown University). Experiment 78 was designed to evaluate if mice cured by chloroquine chemotherapy were as susceptible to rechallenge as mice cured by dietary manipulation of fatty acids and vitamin E.

Experiment 80 was designed to see if mice maintained on a standard mouse chow diet for 2 or 4 weeks prior to infection supplemented with 20% n-3 fatty acids, would be less susceptible to malaria than mice receiving chow alone. These mice had regular vitamin E levels in their diet. This type of dietary approach is similar to one which could be performed in humans in conjunction with chemotherapy to control drug-resistant malaria. A new source of n-3 fatty acids was tested in experiment 81 at concentrations of 2, 4, or 8% of the diet. These diets had restricted levels of vitamin E. Experiment 82

was similar to 80 except here different levels of n-3 fatty acids (5, 10, and 20%) were added to the standard mouse chow diets in order to see if they would render the mice less susceptible to malaria growth. Experiment 83 was similar to experiment 78 where chloroquine was used at different dosages to obtain cures in order to compare its curative to that obtained by dietary manipulation.

In experiment 84 miglyol was added to a standard rodent chow diet with normal levels of vitamin E to see whether it whould interfere with the growth of malarial parasites. This product has been used in humans and could serve as a placebo to n-3 fatty acids in possible future monkey or human dietary experiments to control malaria. Experiment 85 was a repeat of experiment 83. In experiment 86 some of the oxidative byproducts of lipid peroxidation reactions occurring during a malarial infection in mice, whose fatty acid profile was altered to contain more n-3 fatty acids, were quantitated. Experiment 87 was similar to 86 in that some of the oxidative byproducts of lipid peroxidation reactions were measured in mice fed a standard rodent chow diet supplement with different levels of n-3 fatty acids.

RESULTS

MULTIPLE DOSE MODIFIED MM TESTS

Dihydroartemisinin was 100% curative and completely suppressed the parasitemia for 60 days when administered at 100 mg/kg total dose PO for 4 days (D+0, 1, 2, 3 postinfection) at 12 hr intervals to mice injected with a regular MM inoculum. In a similar treatment regimen a 50 mg/kg total dose cured only 1 of 10 mice and exhibited minor suppressive activity (**EXP.46**).

Artesunate (60 mg/kg total dose), Na artelinate (30 and 60 mg/kg total dose) and dihydroartemisinin (240 mg/kg total dose) administered PO for 5 days (D+0, 1, 2,3,4) b.i.d. at 8 hr intervals showed artesunate and Na artelinate were inactive at these levels while dihydroartemisinin cured 9 of 10 mice (EXP.47). Chloroquine administered PO at 140 and 280 mg/kg total dose on days 0 and 1 b.i.d. at 8 hr intervals to drug-sensitive parasites cured all mice. A lower dose of 70 mgs cured 3 of 5 mice while a 35 mgs level cured 1 mouse and extended the survival time of the others. When chloroquine was administered at similar levels to chloroquine-resistant parasites there was 1 cure at 280, and 140, whereas no extension of survival times were noted in the remaining mice (**EXP.59**).

One tetraoxane (BM 07749) cured all mice at 640 and 1280 mg/kg/day when administered PO once a day on D+3, 4, and 5 postinfection. A level of 160 mg/kg/day cured 1 mouse and extended the survival times of the remaining 4 mice. No activity was

observed at 40 mg/kg/day. Two other dimers or trimers of related tetraoxanes tested at similar levels as BM 07749 were inactive (**EXP.61**).

The tetraoxane BM 07749 was retested in experiment 69 along with two other tetraoxanes (BM 07721, BM 07730) all at similar levels and in a similar regimen. BM 07749 cured all mice at the 1280 mg/kg/day level while 1 cure and extension of survival time in the 4 other mice was obtained at 640 mg/kg/day. The 40 and 160 mg/kg/day levels were inactive. BM 07721 cured all mice at 1280 and 640 levels while the 160 dose extended the survival times and the 40 dose level was inactive. BM 07730 extended the survival time at 1280, 640, and 160 levels, while the 40 mg/kg/day dose was inactive.

Compound MA 680, when administered PO on D+3, 4, 5 at 40, 160, and 640 mg/kg/day, did not exhibit any antimalarial activity (**EXP.63**). When administered at higher doses of 2560,5120, and 7680 mg/kg/day in a similar regimen (to experiment 63), still no antimalarial activity was found (**EXP.65**).

TRANSDERMAL ADMINISTRATIONS

Artelinic acid was mixed in a gel containing azone (reported to be enhancing agent) and administered to the hairless skin of mice for either 3 or 6 days b.i.d. at 8 hr intervals commencing on day 0 postinfection. Complete curative activity was found at total doses of 540 mg/kg for 6 days and 270 mg/kg for 3 and 6 days while the 135 mg/kg level cured 4 of 5 mice and extended the survival time of the other mouse (**EXP.48**). Artelinic acid in a similar formulation was administered once a day for either 2,4, or 6 days postinfection. The mice treated for 2 days received a 90 mg/kg total dose which was not active. Mice treated for 4 days receiving a 180 mg/kg total dose produced cures in 4 of 5 mice, with the surviving mouse living twice as long as the controls. A total dose of 270 mg/kg cured all 5 mice (**EXP.49**). In experiment 50 mice were treated with artelinic acid for either 1,2, or 3 days postinfection at 8 hr intervals. Complete curative activity was obtained in the groups treated for 2 or 3 days (180 and 270 mg/kg total dose levels respectively), while only 1 mouse was cured when treated for 1 days with 90 mg/kg total dose. When one group of mice was treated once on day 0 with 45 mg/kg total dose of artelinic acid no antimalarial activity was observed (**EXP.50**).

The next series of experiments compared artelinic acid gel formulations with and without azone. In experiment 53 artelinic acid with azone administered at a total dose of 180 mg/kg for 2 days (D+0, 1) at 8 hr intervals cured 4 of 5 mice with azone and 3 of 5 mice without azone. The same regimen was administered on days 3, 4 postinfection and 1 of 5 mice was cured without azone while no cures were obtained with azone. Extended survival times were observed with both groups.

In experiment 54 artelinic acid administered with or without azone in a total dose of 540 mg/kg for 6 days (starting D+3) b.i.d. at 8 hr intervals cured all mice. When administered for 3 days (commencing on D+3), at a total dose of 270 mg/kg, all mice were cured without azone while only 3 of 5 with azone were cured. In experiment 55 artelinic acid administered with or without azone once on day 0 after infection in a total dose of 40 mg/kg exhibited no antimalarial activity.

When mice were kept like a regular MM test in a room maintained at 75°F for the first 3 days postinfection then switched to a regular 84°F room for the duration of the test a transdermal formulation of artelinic acid with azone (270 mg/kg total dose) administered on days 3, 4, 5 postinfection b.i.d. at 8 hr intervals cured 4 of 5 mice. (**EXP.56**) This was comparable to results obtained when mice were housed in an 84°F room from the day of infection through the duration of the experiment.

In experiment 58 artelinic acid with azone administered at 270 mg/kg total dose to mice on either days 0, 1, and 2 at 8 hr intervals cured all mice as did a similar formulation without azone. When the same formulation was given on days 3, 4, and 5 the gel containing azone cured 4 of 5 mice while the one without azone cured only 3 of 5 mice.

Artelinic acid without azone administered transdermally (10 mg/kg total dose) on days 0 and 1 to mice at 8 hr intervals cured 4 of 5 mice infected with drug-sensitive parasites and 3 of 5 mice infected with chloroquine-resistant parasites (**EXP.60**).

Artelinic acid administered without azone to mice once a day on days 3 and 4 postinfection cured 6 of 10 mice at 360 mg/kg total dose, 3 of 10 mice at 180 mg/kg, and 1 of 10 mice at 90 mg/kg level (**EXP.70**).

In experiment 71 methyl artelinate was as active as artelinic acid in curing mice at total doses of 300 and 150 mg/kg when administered on days 0, 1 and 2 or days 3, 4, and 5 at 8 hr intervals. Five of 5 mice were cured at the 300 mg/kg level on days 0, 1, and 2 and 3, 4, and 5, while the 150 mg/kg dose cured all mice for each compound on days 0,1, and 2 but only 4 of 5 mice were cured when treatment was delayed until days 3,4, and 5 for each compound.

In experiment 72 artemisinin was as active as dihydroartemisinin at a total dose of 300 mg/kg or 150 mg/kg when administered on days 0,1, and 2 at 8 hr intervals (5 of 5 mice were cured for each compound). When treatment was delayed until days 3, 4, and 5 dihydroartemisinin cured 5 of 5 mice at 300 and 150 mg/kg while artemisinin cured only 2 of 5 mice at 300 mg/kg and only extended the survival time of mice at 150 mg/kg.

Cleaning the skin at either 30 minutes, 1 hr or 2 hr after treatment with artelinic acid (without azone) on days 2,3, and 4 postinfection at 8 hr intervals did not interfere with

its curative activity. Whether these mice were cleaned with 70% alcohol, soap and water, or by a gauze wipe all survived when treated with a total dose of 300 mg/kg (EXP.74).

In Balb/c mice (used only this experiment #75) a total dose of 75 mg/kg of either artemisinin, dihydroartemisinin, artelinic acid or methyl artelinate administered on days 3, 4, and 5 postinfection at 8 hr intervals cured 5 of 5 mice. A lower total dose level of 37.5 mg/kg cured all mice given dihydroartemisinin, 3 of 5 mice with artelinic acid, 2 of 5 mice with either methyl artelinate or artemisinin.

Total dose levels of 75 mg/kg for dihydroartemisinin cured 5 of 5 mice when given on days 0,1, and 2 or days 3, 4, and 5 at 8 hr intervals. A 37.5 mg/kg dose cured all mice on days 0, 1, and 2 but only 3 of 5 mice on days 3, 4 and 5. Artemisinin at 75 mg/kg cured 5 of 5 mice when given on days 0,1, and 2 but only 1 of 5 when given on days 3, 4, and 5. Artemisinin at 37.5 mg/kg cured 3 of 5 mice when administered on days 0, 1, and 2 but was inactive if treatment was delayed until days 3, 4, and 5 (EXP.76).

Total dose levels of 75 mg/kg for artelinic acid cured 5 of 5 mice when administered on days 0,1, and 2 at 8 hr intervals, but cured only 1 of 5 mice when treatment was begun on days 3, 4, and 5. A lower total dose level of 37.5 mg/kg was inactive when administered on days 0,1, and 2 or days 3, 4, and 5. Methyl artelinic acid at 75 mg/kg cured 2 of 5 mice when given on days 0, 1, and 2 but was inactive when treatment was delayed until days 3, 4, and 5.

When the mice surviving 60 days postinfection were rechallenged with malaria (experiments 48 through 71) all were susceptible to reinfection and subsequently died except 2 mice in experiment 71 which had parasites present within the 2 weeks prior to rechallenge. These results are in accord with the [illegible]emunition theory of immunity to malaria where a current or recent low level of infection is necessary to maintain a degree of immunity to subsequent challenges.

THOMPSON SUPPRESSIVE AND CURATIVE TEST

The curative results of the 35 tests performed are summarized in Table III.

TABLE III

CURATIVE DATA FOR THOMPSON TESTS

TEST NUMBER	PARASITE LINE	COMPOUNDS TESTED	MG/KG/DAY	NUMBER OF MICE ALIVE DAY 60/TOTAL
673	QHS-R	QHS	160	5/7
			40	3/7
			10	0/7
		Na Artelinate BL 5580	160	7/7
			40	6/7
			10	6/7
		Na Artesunate BL 2855	160	7/7
			40	7/7
			10	4/7
		Chloroquine	160	6/7
			40	7/7
			10	2/7
		CONTROL	0	0/7
674	QHS-R	QHS	160	4/7
			40	2/7
			10	0/7
		Mefloquine	40	7/7
			10	7/7
			2.5	7/7
		Chloroquine	40	5/7
			10	5/7
			2.5	5/7

TABLE III (Cont.)

674	QHS-R	CONTROL	0	0/7
	PV-CR	QHS	160	4/7
			40	4/7
			10	0/7
		Mefloquine	160	5/7
			40	7/7
			10	3/7
		Chloroquine	160	4/7
			40	7/7
			10	6/7
		CONTROL	0	0/7
675	PV-CR	Halofantrine	4	7/7
			1	7/7
			0.25	1/7
		Mefloquine	4	7/7
			1	0/7
			0.25	0/7
		WR 238605	4	6/7
			1	0/7
			0.25	0/7
		Alloxan	100	0/7
			50	0/7
		CONTROL	0	0/7
676	PV-CR	Chloroquine	16	5/7
			4	5/7
			2	0/7

TABLE III (Cont.)

676	PV-CR	Floxacrine	16	3/7
			4	1/7
			2	0/7
		WR 99210	64	7/7
			16	0/7
			4	0/7
			1	
		WR 250417	64	6/7
			16	7/7
			4	0/7
			1	0/7
		CONTROL	0	0/7
677	PV	Chloroquine	128	2/7
			64	1/7
			16	0/7
			2	0/7
		Mefloquine	128	2/7
			64	2/7
			16	0/7
			2	0/7
		Halofantrine	128	6/7
			16	3/7
			2	0/7
			0.5	0/7
		CONTROL	0	0/7
	PV-CR	Chloroquine	128	6/7
			64	7/7
			16	4/7
			2	0/7

TABLE III (Cont.)

677	PV-CR	Mefloquine	128	7/7
			64	7/7
			16	7/7
			2	7/7
		Halofantrine	128	7/7
			16	7/7
			2	7/7
			0.5	4/7
		CONTROL	0	0/7
678	PV	Chloroquine	128	3/7
			64	1/7
			16	0/7
			2	0/7
		WR 238605	64	2/7[T]
			16	7/7
			4	5/7
			1	0/7
		Primaquine	64	4/7
			16	0/7
			4	0/7
			1	0/7
		CONTROL	0	0/7
	PV-CR	Chloroquine	128	7/7
			64	7/7
			16	7/7
			2	0/7
		WR 238605	64	1/7[T]
			16	6/7
			4	7/7
			1	0/7

TABLE III (Cont.)

678	PV-CR	Primaquine	64	5/7
			16	7/7
			4	7/7
			1	0/7
		CONTROL	0	0/7
679	8 AQ-R	WR 238605	64	0/7[T]
			16	7/7
			4	5/7
			2	4/7
		Primaquine	64	7/7
			16	5/7
			4	1/7
			2	0/7
		Chloroquine	64	3/7
			16	5/7
			4	1/7
			2	0/7
		QHS	64	4/7
			16	0/7
			4	0/7
		Halofantrine	64	7/7
			16	7/7
			4	6/7
			2	7/7
		WR 250417	64	6/7
			16	3/7
			4	0/7
			2	0/7
		CONTROL	0	0/7

TABLE III (Cont.)

680	Pyr-R	Proguanil	256	0/7[T]
			64	0/7[T]
		WR 99210	256	2/7
			64	0/7
			16	0/7
			4	0/7
		WR 250417	32	1/7
			16	0/7
			8	0/7
			4	0/7
		Sulfadiazine	4	1/7
			1	0/7
			0.25	0/7
		Pyrimethamine	128	0/7
			64	0/7
		CONTROL	0	0/7
681	PV-CR	Chloroquine	128	6/7
			64	6/7
			16	4/7
			4	6/7
			2	1/7
		Floxacrine	64	6/7
			16	3/7
			4	0/7
		Pyrimethamine	64	6/7
			16	3/7
			4	6/7
			2	4/7

TABLE III (Cont.)

681	PV-CR	Doxycycline	64	5/7
			16	3/7
			4	0/7
		Cycloguanil	128	6/7
			64	6/7
		Quinacrine	128	4/7
			32	6/7
			2	0/7
		Na Artelinate	160	3/7
			40	3/7
			10	4/7
		CONTROL	0	0/7
682	Py	Chloroquine	128	
			64	
			16	
			4	
			1	
		Halofantrine	64	
			16	
			4	
			1	
		Mefloquine	64	
			16	
			4	
			1	
		Sulfadiazine	64	
			16	
			4	
			1	

TABLE III (Cont.)

682	Py	Pyrimethamine	64
			16
			4
			1
		CONTROL	0
683	PV-CR	Chloroquine	128
			64
			16
			4
			1
		Halofantrine	16
			4
			1
			0.25
			0.06
		Cycloguanil	64
			16
			4
			1
		WR 238605	64
			16
			4
			1
		Primaquine	64
			16
			4
			1
		Alloxan	100
		CONTROL	0

TABLE III (Cont.)

684	Pb-CR	Chloroquine	128	0/7
			64	2/7
			16	0/7
			4	3/7
			2	0/4
		Mefloquine	128	7/7
			64	6/7
			16	4/7
			4	6/7
			2	0/7
		CONTROL	0	0/7
685	Pb-MR	Mefloquine	256	1/7
			64	2/7
			16	0/7
			4	0/7
			2	0/7
		Chloroquine	128	0/7
			64	0/7
			16	0/7
			4	0/7
			2	0/7
		Halofantrine	128	4/7
			64	4/7
			16	2/7
			4	2/7
			2	0/7
			0.5	0/7
		QHS	128	0/7
			64	0/7
			16	0/7

TABLE III (Cont.)

685	Pb-MR	Quinine	256	0/7
			64	0/7
			16	0/7
		CONTROL	0	0/7
686	8 AQ-R	WR 238605	128	0/7[T]
			64	0/7
			16	7/7
			4	5/7
			1	0/7
		Primaquine	128	7/7
			64	5/7
			16	4/7
			4	0/7
			1	0/7
		Chloroquine	128	5/7
		CONTROL	0	0/7
	Py	WR 238605	128	0/7[T]
			64	0/7[T]
			16	5/7
			4	4/7
			1	0/7
		Primaquine	128	6/7
			64	6/7
			16	6/7
			4	3/7
			1	0/7

TABLE III (Cont.)

686	Py	Chloroquine	128	4/7
		CONTROL	0	0/7
687	PV*	Chloroquine	64	0/5
			16	0/5
			4	0/5
			2	0/5
		WR 238605	16	0/5[T]
			4	5/5
			1	0/5
			0.25	0/5
		Quinine	256	3/5
			64	0/5
			16	0/5
			4	0/5
		Primaquine	16	0/5
			4	0/5
			1	0/5
			0.25	0/5
		Alloxan	100	0/5
		CONTROL	0	0/7
688	Py	Quinine	2018	1/7
			1024	6/7
			512	4/7
			128	3/7
			32	0/7
			8	0/7

TABLE III (Cont.)

88	Py	WR 99210	64	0/7
			16	0/7
		QHS	32	2/7
			8	0/7
			2	0/7
		Halofantrine	4	6/7
			1	5/7
			0.25	0/7
			0.06	0/7
		CONTROL	0	0/7
		QHS[@]	32	0/7
			8	0/7
			2	0/7
		Halofantrine[@]	4	5/7
			1	1/7
			0.25	0/7
			0.06	0/7
		CONTROL	0	0/7
89	Q-R	Quinine	1024	0/7[T]
			512	5/7
			256	6/7
			64	3/7
		Chloroquine	256	3/7
			32	6/7
			4	1/7
		CONTROL	0	1/7

TABLE III (Cont.)

89	Py	Quinine	1024	1/7[T]
			512	7/7
			256	4/7
			64	2/7
		Quinacrine	128	6/7
			32	4/7
			8	3/7
			2	0/7
		Doxycycline	128	3/7
			32	3/7
			8	0/7
			2	2/7
		Floxacrine	32	6/7
			8	5/7
			2	4/7
		CONTROL	0	0/7
90	Q-R	Quinine	256	5/7
		N-acethylcysteine	64	3/7
		CONTROL	0	0/7
	PV	Quinine	256	0/7
			64	0/7
			16	0/7
		N-acethycysteine	256	0/7
			64	0/7
		CONTROL	0	0/7

TABLE III (Cont.)

2	PV-CR*	Choroquine	128	7/7
			32	5/7
			8	6/7
			2	7/7
		Mefloquine	128	6/7
			32	7/7
			8	7/7
			2	6/7
		Halofantrine	128	7/7
			32	7/7
			8	6/7
			2	7/7
			0.5	3/7
		Quinine	128	7/7
			32	0/7
		CONTROL	0	0/7
)3	8 AQ-R*	Chloroquine	128	5/5
			64	3/5
			16	2/5
			4	1/5
			2	2/5
			1	0/5
		WR 238605	64	0/5[T]
			32	0/5[T]
			16	1/5[T]
			4	5/5
			2	2/5
			1	1/5

TABLE III (Cont.)

693	8 AQ-R*	Primaquine	64	5/5
			32	5/5
			16	5/5
			4	1/5
			2	0/5
			1	0/5
		Mefloquine	128	4/5
			64	4/5
			16	5/5
			4	5/5
			2	4/5
			1	1/5
		Quinine	256	3/5
			64	0/5
			16	2/5
			4	0/5
		CONTROL	0	0/5
694	PV*	Phenylhydrazine	100	0/5
			50	0/5
			25	0/5
		QHS	160	0/5
			40	0/5
			10	0/5
		Na Artelinate	160	5/5
			40	1/5
			10	0/5
		Floxacrine	64	5/5
			16	5/5
			4	0/5

TABLE III (Cont.)

694	PV*	Quinacrine	64	5/5
			16	4/5
			4	0/5
		Alloxan	100	0/5
			50	0/5
			25	0/5
		CONTROL	0	0/5
695	Pb	Bisquinoline	64	0/7
		BM 03796	16	0/7
			4	0/7
		Chloroquine	64	2/7
			16	4/7
			4	0/7
			1	0/7
		Halofantrine	4	3/7
			1	5/7
			0.25	0/7
			0.06	0/7
		CONTROL	0	0/7
	Pb-CR	Bisquinoline	64	2/7
		BM 03796	16	0/7
			4	1/7
		Chloroquine	128	0/7
			64	0/7
			16	2/7
			4	3/7

TABLE III (Cont.)

695	Pb-CR	Halofantrine	128	7/7
			64	6/7
			16	5/7
			4	5/7
		CONTROL	0	4/7
696	Pb*	Na Artelinate	160	5/5
			40	4/5
			10	3/5
			2.5	3/5
		Floxacrine	16	5/5
			4	5/5
			1	0/5
			0.25	0/5
			0.06	0/5
		Mefloquine	64	5/5
			16	5/5
			4	5/5
			1	0/5
			0.25	0/5
		Chloroquine	128	3/5
			64	1/5
			16	0/5
			4	0/5
			2	0/5
			1	0/5
		Halofantrine	64	5/5
			16	5/5
			4	5/5
			2	4/5
			1	1/5
			0.25	0/5
		CONTROL	0	0/5

TABLE III (Cont.)

697	Pb*	Chloroquine	16	2/7
			4	4/7
			2	0/7
		Halofantrine	16	7/7
			4	6/7
			2	3/7
			1	7/7
		Mefloquine	16	7/7
			4	7/7
			2	3/7
			1	1/7
		CONTROL	0	0/7
	Pb-CR*	Chloroquine	128	0/7
			16	0/7
			4	0/7
		Halofantrine	128	6/7
			16	7/7
			4	6/7
			1	6/7
		Mefloquine	128	7/7
			16	7/7
			4	1/7
			1	0/7
		CONTROL	0	0/7

TABLE III (Cont.)

698	Pb	Tetraoxane	1024	7/7
		BM 07749	512	7/7
			256	5/7
			64	2/7
			16	0/7
			4	0/7
		Arteether	1024	7/7
			512	7/7
			256	7/7
			64	7/7
			16	6/7
			4	1/7
		Na Artelinate	1024	4/7
			512	6/7
			256	5/7
			64	0/7
			16	2/7
			4	0/7
		QHS	1024	7/7
			512	7/7
			256	4/7
			64	1/7
			16	1/7
			4	0/7
		CONTROL	0	4/7
699	Pb	Guanylhydrazine	320	2/7
		BL 56390	80	2/7
			20	2/7

TABLE III (Cont.)

699	Pb	Chloroquine	256	3/7
			64	3/7
			16	3/7
			4	2/7
			1	0/7
			0.25	0/7
		Mefloquine	256	7/7
			64	6/7
			16	6/7
			4	5/7
			1	6/7
			0.25	5/7
		Halofantrine	256	7/7
			64	7/7
			16	7/7
			4	4/7
			1	3/7
			0.25	0/7
			0.06	0/7
		CONTROL	0	0/7
701	Pb	Quinine	256	3/7
			64	4/7
			16	0/7
			4	3/7
		Pyrimethamine	256	6/7[T]
			64	4/7
			16	1/7
			4	1/7
			1	4/7
			0.25	6/7

TABLE III (Cont.)

701	Pb	Sulfadoxine	256	7/7
			64	7/7
			16	3/7
			4	1/7
			1	1/7
			0.25	2/7
			0.06	0/7
		Quinacrine	256	7/7
			64	5/7
			16	4/7
			4	0/7
			1	0/7
			0.25	0/7
		CONTROL	0	0/7
702	Pb	Fe chelator BL 59588	320	0/7
			80	3/7
			20	1/7
		WR 158122	256	7/7
			64	7/7
			16	7/7
			4	7/7
			1	6/7
			0.25	4/7
			0.06	5/7
		Doxycycline	256	7/7
			64	6/7
			16	0/7
			4	0/7
			1	2/7
			0.25	0/7

TABLE III (Cont.)

702	Pb	Cycloguanil pamate	256	7/7
			64	7/7
			16	7/7
			4	0/7
			1	0/7
			0.25	0/7
			0.06	0/7
		CONTROL	0	0/7
703	Pb	Tetraoxane BM 07749	4096	3/3
			2048	3/3
			1024	3/3
		QHS	4096	3/3
			2048	3/3
			1024	3/3
		Arteether	4096	0/3[T]
			2048	1/3
			1024	2/3
		CONTROL	0	0/7
704	Pb	Fe chelator BL 58588	320	0/7
			80	0/7
			20	0/7
			5	0/7
		Mefloquine	4	4/7
			1	0/7
			0.25	0/7
			0.06	0/7
			0.02	0/7

TABLE III (Cont.)

704	Pb	WR 158122	1	7/7
			0.25	3/7
			0.06	0/7
			0.02	0/7
		Doxycycline	4	0/7
			1	0/7
			0.25	0/7
			0.06	0/7
			0.02	0/7
		Pyrimethamine	1	0/7
			0.25	0/7
			0.06	0/7
			0.02	0/7
		CONTROL	0	0/7
705	Pb	Bisquinoline BM 03821	1024	7/7
			512	7/7
			256	7/7
			64	7/7
			16	5/7
			4	3/7
		Bisquinoline BL 57511	1024	$0/7^{T}$
			512	$0/7^{T}$
			256	$6/7^{T}$
			64	0/7
			16	3/7
			4	0/7
		Chloroquine	1024	$0/7^{T}$
			512	0/7
			256	5/7
			64	5/7
			16	0/7
			4	2/7

TABLE III (Cont.)

705	Pb	CONTROL	0	2/7
		Bisquinoline BM 03821	1024	7/7
			512	7/7
			256	7/7
			64	7/7
			16	2/7
			4	0/7
		Bisquinoline BL 57511	1024	7/7
			512	5/7
			256	6/7
			64	3/7
			16	0/7
			4	0/7
		Chloroquine	1024	0/7[T]
			512	0/7[T]
			256	0/7[T]
			64	4/7
			16	2/7
			4	1/6
		CONTROL	0	0/7
706	PV	Chloroquine	32	0/7
			8	0/7
			2	0/7
		Mefloquine	32	4/7
			8	1/7
			2	0/7
		Halofantrine	8	5/7
			2	0/7
			0.5	0/7

TABLE III (Cont.)

706	PV	CONTROL	0	0/7
	PV-CR	Chloroquine	128	2/7
			32	3/7
			8	2/7
			2	0/7
		Mefloquine	128	4/7
			2	0/7
		Halofantrine	128	7/7
			32	6/7
			8	7/7
			2	4/7
			0.5	0/7
		CONTROL	0	0/7
707	8 AQ-R	WR 238605	64	3/7[T]
			16	7/7
			4	3/7
			1	0/7
		Primaquine	64	6/7
			16	5/7
			4	3/7
			1	0/7
		Chloroquine	64	4/7
			16	4/7
			4	3/7
			1	0/7
		Mefloquine	64	7/7
			16	6/7
			4	5/7
			1	0/7

TABLE III (Cont.)

707	8 AQ-R	Halofantrine	64	7/7
			16	7/7
			4	5/7
			1	2/7
		Quinine	64	0/7
			16	0/7
			4	0/7
		CONTROL	0	0/7
708	MM	Chloroquine	1024	$0/7^{T}$
			512	$1/7^{T}$
			256	7/7
			64	1/7
			16	0/7
			4	0/7
			2	0/7
		Mefloquine	64	3/7
			16	3/7
			4	0/7
			1	0/7
		Halofantrine	64	4/7
			16	5/7
			4	1/7
			1	0/7
			0.25	0/7
		Quinine	64	0/7
			16	0/7
			4	0/7
		WR 238605	64	$1/7^{T}$
			16	7/7
			4	2/7
			1	0/7

TABLE III (Cont.)

708	MM	CONTROL	0	0/7
709	Py-NL	Chloroquine	128	7/7
			64	7/7
			32	7/7
			16	7/7
			4	7/7
			2	7/7
			1	7/7
		Halofantrine	16	7/7
			4	7/7
			1	7/7
			0.25	7/7
		CONTROL	0	7/7
	Py	Chloroquine	128	3/7
			64	1/7
			32	1/7
			16	2/7
			4	3/7
			2	0/7
			1	0/7
		Halofantrine	16	6/7
			4	3/7
			1	3/7
			0.25	0/7
		CONTROL	0	0/7

* = Balb/c mice were used

T = Toxic drug level

SYNERGISTIC STUDIES

WR 238417, the putative biguanite precursor of a triazine (WR 99210), exhibited synergistic suppressive antimalarial activity with sulfadiazine at both a 4:1 and 8:1 ratio respectively against pyrimethamine-resistant parasites. Synergistic activity was also observed when WR 250417 was administered with pyrimethamine in an 8:1 ratio respectively, against pyrimethamine-resistant parasites.

N-acetylcysteine did not increase the toxicity of quinine when both were administered at 48 mg/kg once IV on day 3 postinfection against quinine-resistant parasites. Synergistic activity was observed between N-acetylcysteine and quinine when administered orally for 3 days against drug-sensitive *P. vinckei* parasites.

LOUDERBACK STERILIZING MEDIUM

A regular MM inoculum of $6X10^5$ parasitized erythrocytes mixed in LSM for 30 minutes killed all the drug-sensitive parasites as judged by inoculating the blood into mice and observing no parasite growth (**EXP.62 - TABLE IV**).

In experiment 64, using drug-sensitive parasites, inoculums of $6X10^5$ (1X) and 5X were sterilized with LSM while a 10X inoculum still retained a few parasites since 1 of 4 mice developed an infection (**TABLE IV**). In experiment 68 a 10X inoculum of drug-sensitive parasites was sterilized with LSM when administered SC or IV to mice (**TABLE IV**). Inoculum levels of 100X and 1000X sterilized drug-sensitive parasites while an inoculum of 3000X was not sterilized (**EXP.73 - TABLE IV**). In experiment 79 LSM did not sterilize inoculum levels of 100X, 1000X, or 5000X when tested against drug-sensitive parasites (**TABLE IV**).

Qinghaosu-resistant parasites at levels of 1X, 100X, 1000X, and 5000X were not killed by LSM. These parasites reside primarily within reticulocytes compared with drug-sensitive parasites which invade mature red blood cells (**TABLE V**).

TABLE IV

CURATIVE EFFECT OF LOUDERBACK STERILIZNG MEDIUM OF DRUG-SENSITIVE *PLASMODIUM BERGHEI* PARASITES

EXP. NUMBER	GROUP NUMBER	PARASITE INOCULUM	BLOOD TREATED WITH LSM	NUMBER MICE DEAD/ DAY DIED	NUMBER MICE ALIVE DAY 60/ TOTAL
62	1	1X[@]	NO	3/6 5/7 2/8	0/10
	2	1X	YES	0	10/10
64	1	1X	NO	3/6 2/7	0/5
	2	1X	YES	0	5/5
	3	5X	NO	1/5 2/6 1/7 1/8	0/5
	4	5X	NO	4/6 1/7	0/5
	5	5X	YES	0	5/5
	6	5X	YES	0	5/5
	7	10X	NO	1/5 4/6	0/5
	8	10X	NO	1/5 1/6 3/7	0/5
	9	10X	YES	0	5/5
	10	10X	YES	1/10	4/5
68	1	10X	NO	5/6 4/7 1/9	0/10
	2	10X	YES	0	15/15
	3*	10X	NO	10/6	0/10
	4*	10X	YES	O	10/10
73	1	100X	NO	2/6 1/7 2/8	0/5
	2	100X	YES	0	5/5
	3	1000X	NO	2/4 1/5 2/7	0/5
	4	1000X	YES	O	5/5
	5	5000X	NO	2/4 1/5 1/6	0/4
	6	5000X	YES	2/9 1/10	1/4
79	1	100X	NO	4/4 1/5	0/5
	2	100X	YES	2/6 2/9 1/48	0/5
	3	1000X	NO	3/4 2/6	0/5
	4	1000X	YES	1/4 1/5 1/8	2/5
	5	5000X	NO	2/3 3/4	0/5
	6	5000X	YES	2/4 1/7 1/10 1/15	0/5

[@] X = Regular MM inoculum of $6X10^5$ parasitized RBC's
* = IV route of parasite inoculation.

TABLE V

CURATIVE EFFECT OF LOUDERBACK STERILIZING MEDIUM ON QINGHAOSU-RESISTANT *PLASMODIUM YOELII* PARASITES

EXP. NUMBER	GROUP NUMBER	PARASITE INOCULUM	BLOOD TREATED WITH LSM	NUMBER NUMBER MICE DEAD/ DAY DIED	MICE ALIVE DAY 60/ TOTAL
77	1	1X	NO	1/4 1/6 1/7 2/9	0/5
	2	1X	YES	1/7 1/22	3/5
	3	100X	NO	3/4 1/7 1/8	0/5
	4	100X	YES	1/4 3/7 1/9	0/5
	5	1000X	NO	1/3 3/4 1/7	0/5
	6	1000X	YES	1/4 1/6 1/7 1/9	1/5
	7	5000X	NO	4/3 1/7	0/5
	8	5000X	YES	1/4 2/6	2/5

RATE OF PARASITE CLEARANCE STUDIES

Qinghaosu administered SC once on day 3 postinfection at 640 or 160 mg/kg significantly reduced the parasitemia by 35 hrs post-treatment. By day 3 parasite were not observed at the 640 mg/kg level but had recrudesced by day 10 post-treatment. The 160 mg/kg dose did not clear the parasites at anytime. Chloroquine at 160 and 80 mg/kg reduced parasite members by 35 hrs post-treatment. No parasites were observed by 58 hrs and remained negative until day 5 post-treatment when parasites recrudesced. The 80 mg/kg level of chloroquine did not clear the parasites at anytime.

MAINTENANCE OF WR 238605-RESISTANT LINE THROUGH MOSQUITOES

This drug-resistant line has remained stable while maintained under weekly drug pressure of 32 mg/kg/day. The drug pressure can not be increased because the next highest level of 64 mg/kg/day is toxic for the mice.

There is no change in cross resistance patterns to standard antimalarials. The parent drug-sensitive line also has remained susceptible to standard antimalarial agents.

ANTIOXIDANT STUDIES INVOLVING CHANGING FATTY ACID PROFILES IN MEMBRANES

In experiment 73 mice fed a vitamin E restricted diet containing an ethyl ester concentrate of menhaden oil (our normal n-3 fatty acid oil) for 1 month prior to infection and continued for only 1 week post-infection exhibited marked suppressed parasitemias and increased survival levels.

Balb/c mice reacted in a similar manner as CD-1 (our regular mouse type) to the vitamin E and or PABA restricted diets containing n-3 fatty acids. (EXP.74). The parasitemia was completely suppressed in 7 of 9 mice fed the double deficient diet (without vitamin E or PABA) and 9 of 10 mice fed the diet containing vitamin E but not PABA. Survival rates were 100% in both of these groups.

Since we normally start most diet experiments with weanling mice experiment 75 was performed to evaluate whether starting the vitamin E restricted n-3 fatty acid diet 1 week after young mice were fed chow and then followed for 2 or 4 weeks. The vitamin E levels in mice were similarly low whether started at 3 weeks of age or 4 weeks of age.

In experiment 76 the antioxidant coenzyme Q_{10} (ubiquinone 50) administered PO at 100, 36 or 3 mg/kg/day for 7 consecutive days, to mice maintained on a n-3 diet deficient in vitamin E, did not interfere with the diets antimalarial effect. Therefore coenzyme Q cannot substitute for vitamin E as an antioxidant during a malarial infection.

In experiment 77 the various antibody isotype levels were followed at weekly intervals after the primary and secondary infection in mice maintained on the n-3 diet without vitamin E. The various isotypes of IgG antibodies did vary during both the primary and secondary infection. This experiment is still continuing and the data has not been analyzed.

In experiment 78 MCT oil in a vitamin E restricted diet did not interfere with the growth of malarial parasites. Therefore this oil could be used as a placebo oil in future studies in monkeys. Also in this experiment mice cured with chloroquine were compared with diet-cured mice as to their susceptibility to reinfection. Preliminary results suggest both chloroquine-cured mice and diet-cured mice are refractory to challenge.

The results of experiment 80 showed mice maintained on a standard mouse chow diet for either 2 or 4 weeks prior to infection (with normal vitamin E levels) supplemented with 20% n-3 fatty acids suppressed the parasitemia and produced some curative activity.

In experiment 81 a new type of n-3 fatty acids fed at 2, 4 or 8% of the diet suppressed and cured the mice in a similar fashion to the regular highly purified n-3 fatty acids we get from NIH.

In experiment 82 the addition of either 5, 10 or 20% n-3 fatty acids to a standard chow diet containing normal vitamin E levels did suppress the parasite growth in each level and increased their survival times.

Experiment 83 is still in progress and the rechallenge of the mice cured by diet compared with those cured by chloroquine will be completed within the next few months.

In searching for the best placebo to use with n-3 fatty acid in a supplementation study, miglyol oil and MCT oil were tested and both did not interfere with the growth of malarial parasites when given as a 20% supplement to normal mouse chow while 20% n-3 fatty acids did retard the growth of malaria. Therefore both miglyol and MCT oil could serve as a control to n-3 fatty acids in a monkey supplementation study using human malaria (**EXP.84**).

When experiment 84 was repeated (**EXP.85**) miglyol and MCT oil did not interfere with malarial when added to a standard mouse chow diet containing normal levels of vitamin E. These two experiments were done in mice infected with drug-sensitive *P. yoelii.*

In experiment 86 mice on a vitamin E restricted diet containing 4% n-3 fatty acid exhibited enhanced levels of lipid peroxidation byproducts in their urine. The two parameters measured were malonaldehyde and thiobarbituate active substances.

In experiment 87 mice on a standard mouse chow diet supplemented with either 5, 10, or 20% n-3 fatty acids also exhibited some increase in the levels of lipid peroxidation products in their urine. Both of these two experiments will be followed for the next month to obtain data through the parasitemia episode.

CONCLUSIONS

New novel chemicals active against malaria are needed to combat drug-resistant parasites. The 39 active compounds identified this year in the primary MM test need to be evaluated in a secondary battery of tests to determine which ones are worthy to possibly test against human malaria in monkeys. In order to find the best compound in a lead directed synthesis program more refined testing such as multiple dosing experiments against drug-sensitive and drug-resistant parasites has been done with analogs of qinghaosu, tetroxanes and bisquinolines. Dihydroartemisinin is the most active qinghaosu analog, BM 07749 is the most active tetroxane, and BM 03821 is the most active bisquinoline against drug-sensitive parasites. More testing is planned to test these compounds by SC vs PO routes and against drug-resistant parasites. Extensive testing with WR 238605 showed delayed toxicity was encountered at 64 mg/kg/day for 3 days.

Transdermal application of qinghaosu analogs showed dihydroartemisinin was more active than artelinic acid, methyl artelinate and artemisinin. This novel method of drug delivery will be further used to evaluated other qinghaosu analogs as well as other chemical types.

Synergistic studies showed WR 238417, the putative biguanide precursor of a triazine WR 99210, acted synergistically with pyrimethamine and sulfadiazine against pyrimethamine-resistant parasites. More synergism studies are planned to detect novel drug interactions.

A new blood sterilizing medium (Louderback Sterilizing Medium) was incubated *in vitro* with parasitized RBC's and found to kill malarial parasites as judged by inoculating the treated parasites into mice and observing no growth. Higher levels of parasites tested with this medium were not completely killed, so experiments are continuing to explain this phenomenon.

The WR 238605-resistant line was continuously passed through mosquitoes to maintain a gametocyte population and monitor any changes in drug-sensitivity. Similar experiments will continue to mimic as closely as possible what is occurring in nature with parasites being continuously passed in humans.

In a series of antioxidant studies malaria could be controlled in mice fed a normal mouse chow diet supplemented with 5, 10 or 20% omega-3 fish oil. This is an important finding since the vitamin E levels were normal in the diet, therefore, malaria maybe controlled by supplementation of omega-3 fatty acids to malaria patients. We are currently testing this supplementation experiment in normal monkeys for tolerance studies and if successful envision trying it in monkeys infected with human malaria then into humans

with drug-resistant malaria. We envision this approach to be used in conjunction w. chemotherapy.

In the next year we plan on adding two new phases to our program, one involving *in vitro* evaluation of compounds against *P. falciparum* and the second being an *in vivo* cerebral malaria model in mice to find new compounds active against parasites in the central nervous system.

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